Langerhans Cell Migration into Ultraviolet Light-Induced Squamous Skin Tumors Is Unrelated to Anti-Tumor Immunity

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There has been much speculation as to the role of Langerhans cells (LC) in the induction of anti-tumor immunity. Whereas there is considerable circumstantial evidence that disruptions in the density and function of these cells during the early stages of ultraviolet (UV) light- and chemical carcinogen-induced carcinogenesis may be important for enabling developing neoplasms to escape immune destruction, the role of the large number of these cells found infiltrating developed skin tumors is less clear. To investigate this we have compared the LC density infiltrating transplanted non-immunogenic and immunogenic UV-induced murine tumors as well as LC in the epidermis overlying the tumors. Whereas two non-immunogenic tumor lines attracted large numbers of Ia^+ dendritic cells, an immunogenic tumor line did not. Similar results were obtained whether the tumors were transplanted into syngeneic immunocompetent or athymic immunodeficient mice. Hence, there was no relationship between tumor immunogenicity or host immunocompetence and Ia^+ dendritic cell density. Furthermore, there was no correlation with the pattern of T-cell infiltration of the tumors or CD4/CD8 cell ratio. Our results also indicate that whereas UV light decreased Ia^+ cell density, both in the epidermis and the tumors, it did not inhibit the tumors from attracting Ia^+ dendritic cells. Thus, the Ia^+ dendritic cells infiltrating skin tumors are unlikely to indicate a host immune response to the tumor, but are more likely to be attracted by tumor-derived cytokines. *J Invest Dermatol 97:830–834, 1991*

Langerhans cells (LC) are epidermal dendritic cells that express high levels of class II major histocompatibility complex (MHC)-encoded glycoproteins (Ia in mouse, HLA-DR in humans). They are an important component of the skin immune system, being essential for the initiation of immune responses against cutaneous antigens. LC take up antigen in the epidermis and then migrate with the antigen to local lymph nodes where they present this antigen to T lymphocytes, resulting in T-cell activation [1].

Because ultraviolet (UV) light is the prime etiologic event responsible for skin cancer in man [2], and UV-induced skin cancers have been shown to be immunogenic in mice [3], it is likely that LC, as a key component of the skin immune system, may be important in the pathogenesis of skin cancer.

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Abbreviations:
ANOVA: one-way analysis of variance
LC: Langerhans cells
MHC: major histocompatibility complex
N: number of mice in group
No UV: unirradiated mice
ns: not significantly different
UV: ultraviolet light
UV-cont: mice irradiated with UV light for 4 weeks prior to inoculation with the tumor cell line with irradiation continuing during the 4 weeks of tumor growth
UV-term: mice irradiated with UV light for 4 weeks prior to inoculation with the tumor cell line

UV light depletes LC from murine skin [4,5], resulting in reduced contact sensitivity responses [6] due to suppressor lymphocyte activation [7]. However, the role of these UV-induced changes in local Ia^+ epidermal dendritic cells during UV-induced carcinogenesis remains unclear because UV light not only induces local but also systemic immunosuppression. Skin tumor cells injected intravenously into UV-irradiated mice have been shown to grow in the lungs [8] and contact sensitizers applied to skin distant to the UV irradiation site also induce immunosuppression [9] despite the LC density at this distant site being normal [10]. However, we have shown that during the promotor phase of chemical carcinogenesis, the density of epidermal Ia^+ dendritic cells is also decreased [11,12], resulting in local but not systemic immunosuppression [13,14]. This suggests that the reduction in LC during the early stages of carcinogenesis may allow neoplastically transformed cells to activate suppressor lymphocytes and thus escape from immune elimination.

It has also been observed that spontaneous human [15,16] as well as chemically induced murine [12] tumors are infiltrated with large numbers of LC, the role of which is unclear. In the present study we have examined the LC density in UV-induced tumor lines that are highly immunogenic or of low immunogenicity, in both immunocompetent and immunoincompetent athymic mice. We have also investigated the effects of UV irradiation on the LC density in order to determine whether the large number of LC-infiltrating skin tumors is of functional importance for the immune response against the tumors.

MATERIALS AND METHODS

Animals Inbred female Skh:HR-1 mice were bred and housed in the Veterinary Pathology Animal House of the University of Sydney. BALB/c athymic mice were obtained from the Animal Resources Centre, Perth, Australia. Mice were 6–8 weeks old at the

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commencement of the experiments and were used with approval from the University of Sydney Animal Ethics Committee.

Squamous Cell Carcinoma Cell Lines Squamous cell carcinomas were induced in inbred Skh : HR-1 mice by chronic minimally erythemal UV irradiation. These tumors were established and maintained in culture as cell lines grown in Dulbecco's Modification of Eagles Medium (Cytosystems, Castle Hill, N.S.W., Australia) supplemented with 10% newborn calf serum (Cytosystems). Three squamous cell carcinoma lines were used in these experiments: T7 and T89, weakly or non-immunogenic lines that grow when transplanted into both unirradiated and UV-irradiated host mice; T79, a highly immunogenic line that does not grow when transplanted into normal syngeneic host mice, but only in UV-irradiated or athymic hosts.

For inoculation into mice, the cells were recovered by treatment with phosphate-buffered saline (PBS, pH 7.3) containing 0.25% trypsin and 0.4% tetrasodium ethylenediaminetetraacetic acid. The cells were washed twice in PBS and 2×10^5 cells injected intradermally into the central upper region of the dorsal trunk.

Ultraviolet (UV) Light Irradiation Simulated solar UV light was provided by a bank of fluorescent tubes (6 × Sylvania F40BL UVA tubes and a single Oliphant FL40SE UVB tube) and filtered through cellulose triacetate (Kodacel 0.125 mm, Eastman, Rochester, NY, USA) to remove UVC [17]. Mice were exposed 5 times per week to a minimal erythemal dose of UV light (0.162 J/cm² UVB and 3.12 J/cm² UVA per day), which was increased by 20% each week for up to 8 weeks of irradiation.

Two different radiation regimes were used. UV terminating: groups of mice were irradiated with UV light for 4 weeks, and then the following day inoculated with one of the tumor cell lines, mice received no further irradiation and were killed 4 weeks following injection of the tumor cells. UV continuing: groups of mice were irradiated with UV light for 4 weeks, inoculated with cells from a tumor line, and irradiation was continued for a further 4 weeks, mice were then killed upon completion of the irradiation period.

Preparation and Staining of Tissues On completion of the UV irradiation protocols the mice were killed by cervical dislocation. The tumors were removed, snap frozen in liquid nitrogen, and stored at −80° for immunohistochemical analysis. The skin immediately above and from 1–5 mm adjacent to the tumors was surgically removed for preparation of epidermal sheets and immunohistochemical staining as we have described previously [14]. The epidermal sheets were fixed in acetone and then incubated overnight at 4° with rat anti-Ia monoclonal antibody-containing supernatant from the TIB 120 cell line (American Type Culture Collection, MD, USA). The sheets were then stained using indirect immunoalkaline phosphatase with biotin-conjugated goat anti-rat IgG and streptavidin-conjugated alkaline phosphatase (Caltag, San Francisco, CA, USA) [18]. The stained sheets were then mounted on glass microscope slides.

For immunohistochemical staining of the tumor material, 6 μ frozen sections were cut using a cryostat onto gelatin-coated glass slides and stained using the biotin-streptavidin indirect immunoalkaline phosphatase method. Cell culture supernatants containing the following primary monoclonal antibodies, as previously described [19] were used: rat anti-Ia, rat anti-Thy-1 (T-24.31.7), rat anti-L3T4 ($CD4^+$ cells, GK1.5), or rat anti-Lyt-2 ($CD8^+$ cells, 31M).

Stained cells were visualized by light microscopy and their number counted manually. The area of the epidermal sheet or tumor section was determined using a microcomputer-based video image analysis system (Chromatic, Wild-Leitz, Australia).

Statistical Analysis Multiple group analysis within an experiment for Ia^+ epidermal and tumor-infiltrating cells as well as for Thy-1^+ tumor-infiltrating cells was by one-way analysis of variance (ANOVA). The CD4/CD8 ratio was not normally distributed and hence was analyzed by the Mann-Whitney U test. A p value of <0.05 was regarded as significant.

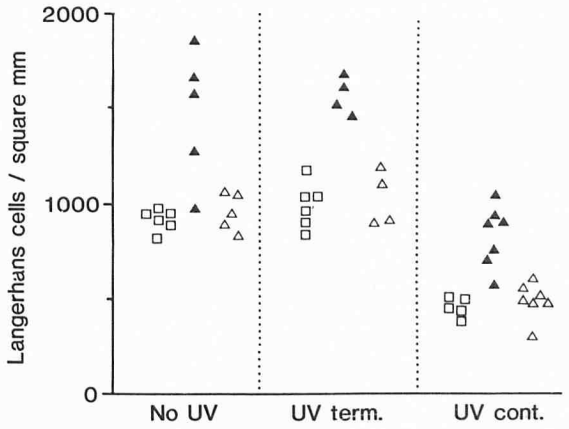

Figure 1. Langerhans cell density in epidermis of Skh : HR-1 mice that had not been injected with tumor cells (□), and epidermis above (▲) and adjacent (△) to the transplantable tumor line T89. Mice were either unirradiated (no UV), irradiated with UV light for 4 weeks prior to inoculation with the T89 cell line (UV-term), or were irradiated with UV light for 4 weeks prior to inoculation with T89 cells with irradiation continuing during the 4 weeks of tumor growth (UV-cont). Each *point* represents a single mouse.

RESULTS

Effects of Tumors on Epidermal Langerhans Cell Density The T89 squamous carcinoma cell line (of low immunogenicity) transplanted into mice increased the density of Ia^+ dendritic cells above but not adjacent to the tumor (Fig 1). There was a significantly increased LC density in epidermis above the tumor compared to the density in both unirradiated mice without a tumor and epidermis adjacent to the tumor (each $p < 0.001$, ANOVA, F stat for equal means: 30.82 df 8,40). In mice irradiated for 4 weeks prior to tumor transplantation, but who received no further irradiation following inoculation with T89 cells (UV-term), there was no significant difference in LC density from unirradiated mice for any of the epidermal areas. The epidermis above the tumor retained a significantly higher LC density than either the epidermis of mice without a tumor or epidermis adjacent to the tumor (both $p < 0.001$). For mice irradiated with UV light for 4 weeks prior to tumor transplantation with the irradiation being continued during the 4 weeks of tumor growth (UV-cont), the density of epidermal Ia^+ dendritic cells was significantly lower than each respective epidermal region of unirradiated mice ($p < 0.001$ for each region) or mice that were only irradiated up to the time of tumor inoculation ($p < 0.001$ for each region). However, the LC density above but not adjacent to the tumor was significantly higher than the LC density in the epidermis of mice without a tumor ($p < 0.001$).

When another squamous carcinoma cell line of low immunogenicity, T7, was inoculated into Skh : HR-1 mice, similar results were obtained (Table I; ANOVA F stat for equal means: 28.23 df 8,54). There was a significant increase in LC density above but not adjacent to the tumor compared to mice without a tumor. When mice received UV term, there was still a significant increase in LC density above the tumor compared to epidermis adjacent to the tumor or to mice without a tumor. In mice irradiated with UV-cont the density of epidermal Ia^+ dendritic cells was significantly decreased for all epidermal regions. However, the density above but not adjacent to the tumor was significantly higher than the epidermis of mice without a tumor.

In contrast, the highly immunogenic tumor T79, which will not grow in unirradiated mice, did not attract LC into the epidermis above the tumor (Table I; ANOVA F stat for equal means: 52.994 df 7,35). The area above the site of T79 inoculation into unirradiated mice contained an LC density that did not differ from that of mice that were not injected with tumor cells. In mice irradiated with UV-term, the LC density above and adjacent to the tumors was significantly reduced compared to unirradiated mice. There was no

Table I. Langerhans Cells in Epidermis Above and Adjacent to T7 and T79 Tumors 4 Weeks Following Inoculation of Tumor Cells into UV Irradiated and Unirradiated Skh:HR-1 Mice

Tumor Line	UV Radiation	Epidermal Region[a]	Mean (SD)	N	p[b]	p[c]	p[d]	p[e]
T7	No UV	No tumor	886 (55)	6				
T7	No UV	Above	1326 (284)	8	<0.001			
T7	No UV	Adjacent	692 (118)	8	ns		<0.001	
T7	UV-term	No tumor	952 (117)	6		ns		
T7	UV-term	Above	1316 (274)	7	<0.01	ns		
T7	UV-term	Adjacent	285 (305)	7	<0.001	<0.001	<0.001	
T7	UV-cont	No tumor	427 (45)	5		<0.001		<0.001
T7	UV-cont	Above	916 (159)	8	<0.001	<0.001		<0.001
T7	UV-cont	Adjacent	320 (159)	8	ns	<0.001	<0.05	ns
T79	No UV	No tumor	886 (55)	6				
T79	No UV	Above[f]	883 (34)	6	ns			
T79	UV-term	No tumor	952 (117)	6		ns		
T79	UV-term	Above	687 (49)	6	<0.001	<0.01		
T79	UV-term	Adjacent	744 (73)	6	<0.001		ns	
T79	UV-cont	No tumor	427 (45)	5		<0.001		<0.001
T79	UV-cont	Above	413 (94)	4	ns	<0.001		<0.001
T79	UV-cont	Adjacent	429 (44)	4	ns		ns	<0.001

[a] Langerhans cells in epidermis from mice that were not inoculated with tumor (no tumor), or from epidermis above the tumor (above), or from epidermis adjacent to the tumor (adjacent).
[b] Statistical comparison to mice with no tumor (ANOVA).
[c] Statistical comparison with unirradiated mice (ANOVA).
[d] Statistical comparison of epidermis above and adjacent to the tumors (ANOVA).
[e] Statistical comparison of UV terminating with UV continuing (ANOVA).
[f] T79 tumor does not grow in unirradiated mice, hence epidermis taken from above inoculation site.

difference in LC density above and adjacent to the tumor. In mice that received UV-cont there was no significant difference in LC density above or adjacent to the tumor compared to mice that did not receive the tumor.

The tumor lines had a similar effect on LC density when transplanted into athymic mice (Fig 2). Both the T89 and T7 tumors attracted significantly increased numbers of LC into both the epidermis above ($p < 0.001$) and adjacent ($p < 0.001$) to the tumors compared to the epidermis of mice without a tumor (ANOVA; F stat for equal means: 44.212 df 6,25). There was a significantly larger number of LC above than adjacent to the tumors ($p < 0.001$). In contrast, T79 did not attract LC, as there was no significant difference in LC density either above or adjacent to the tumor compared to epidermis from mice without a tumor.

Ia$^+$ Cells Infiltrating Tumors T89, but not T7 or T79 tumors, growing in Skh:HR-1 mice displayed extensive Ia staining, making it impossible to quantitate infiltrating Ia$^+$ cells. There were large numbers of Ia$^+$ cells infiltrating T7 tumors, and whereas UV-term had no effect on the density of these cells, UV-cont significantly decreased their density (Table II; ANOVA F stat for equal means: 39.777 df 4,28). The immunogenic tumor T79 attracted significantly lower numbers of Ia$^+$ cells than did T7, and UV-cont reduced the density of these infiltrating cells.

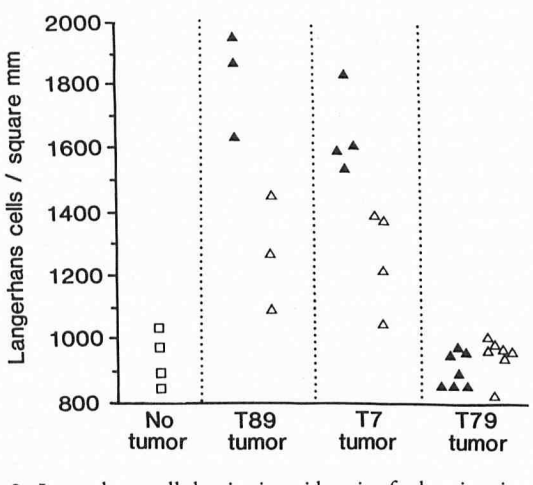

Figure 2. Langerhans cell density in epidermis of athymic mice that had not been injected with tumor cells (□), and epidermis above (▲) and adjacent (△) to the transplantable tumor lines T89, T7 and T79. Each *point* represents a single mouse.

The T89 tumor did not express Ia when growing in athymic mice, and attracted large numbers of Ia$^+$ cells, as did T7 (Table II; ANOVA F stat for equal means: 13.88 df 2,11). In contrast, T79 attracted significantly fewer Ia$^+$ cells than either the T89 or T7 tumors.

Thy-1$^+$ Cells Infiltrating Tumors T89 tumors growing in Skh:HR-1 mice also expressed Thy-1, making it impossible to quantitate Thy-1$^+$ infiltrating cells. The number of Thy-1–expressing cells infiltrating T7 tumors was quite high, and UV-cont but not UV-term significantly increased the density of these cells (Table III; ANOVA F stat for equal means: 22.823 df 4,28). T79 tumors contained significantly fewer Thy-1$^+$ infiltrating cells, and, whereas UV-cont increased the mean number of these cells compared to UV-term, this was not significant.

There was a small number of Thy-1$^+$ cells infiltrating T89 tumors growing in athymic mice. T7 attracted significantly larger numbers of these cells, and T79 tumors contained an intermediate number (Table III; ANOVA F stat for equal means: 13.88 df 2,11).

Ratio of CD4- to CD8-Positive Cells Infiltrating Tumors T89 was neither CD4- nor CD8-positive when growing in Skh:HR-1 mice. All three tumors contained large numbers of CD4- and CD8-positive infiltrating cells. There was a significantly higher ratio of CD4 to CD8 cells in T79 but not T7 compared to T89 tumors ($p < 0.05$, Table IV). Neither UV irradiation protocol effected this ratio. None of these tumors transplanted into athymic mice contained detectable levels of CD4- or CD8-positive cells.

DISCUSSION

We have examined whether the density of Ia$^+$ dendritic cells infiltrating murine UV-induced tumors is related to the immunogenicity of the tumor or the immunocompetence of the host mice, and how UV irradiation affects these cells. There was no relationship between tumor immunogenicity or host immunocompetence and the ability of these tumors to attract LC. Furthermore, even in the presence of continual UV irradiation, which depletes the density of these cells, the tumors were able to attract large numbers of LC. This suggests that the attraction of LC to skin tumors is independent

Table II. Ia$^+$ Cells Infiltrating Tumors 4 Weeks Following Inoculation into UV Irradiated and Unirradiated Skh:HR-1 or Unirradiated Athymic Mice

Tumor Line	Mice	UV Radiation	Mean (SD)	N	p[a]	p[b]	p[c]	p[d]
T7	Skh:HR-1	No UV	286 (26)	8				
T7	Skh:HR-1	UV-term	286 (62)	8	ns			
T7	Skh:HR-1	UV-cont	231 (33)	8	<0.01	<0.01		
T79	Skh:HR-1	UV-term	114 (10)	5			<0.001	
T79	Skh:HR-1	UV-cont	54 (24)	4		<0.05	<0.001	
T89	Athymic	No UV	388 (90)	3			ns	
T7	Athymic	No UV	341 (29)	4				
T79	Athymic	No UV	167 (62)	7			<0.001	<0.001

[a] Statistical comparison with unirradiated mice (ANOVA).
[b] Statistical comparison of UV terminating with UV continuing (ANOVA).
[c] Statistical comparison with T7 tumor line (ANOVA).
[d] Statistical comparison with T89 tumor line (ANOVA).

of the host immune response to the tumor and is possibly due to production of soluble factors that are chemoattractive for LC.

Many murine UV-induced skin tumors are highly immunogenic, and are immunologically rejected when transplanted into normal syngeneic hosts. These tumors will, however, grow when transplanted into mice that have been immunosuppressed with UV light [3]. We have compared the ability of two non-immunogenic (T89 and T7), and an immunogenic (T79) UV-induced squamous cell carcinoma cell line to attract LC. LC infiltration of murine tumors can only be identified by immunohistochemical staining and their dendritic morphology. As their staining pattern is not unique outside of the epidermis, and their dendritic morphology is not always obvious in frozen sections, LC in the epidermis above the tumors were quantitated to give more accurate results.

Both non-immunogenic tumor lines increased the density of LC in the epidermis overlying tumors transplanted into either syngeneic immunocompetent or immunodeficient athymic mice. In contrast, the immunogenic tumor line did not increase the number of epidermal LC in either host. This lack of a relationship between tumor immunogenicity, or host immunocompetence, and ability to attract LC indicates that the increased density of these cells did not result from a host immune response.

To enable the immunogenic tumor to grow, it was necessary to irradiate the host mice with UV light, which depletes the LC density. During the 4 weeks of tumor growth in the absence of further irradiation, the LC density recovered. The increased epidermal LC density above the non-immunogenic tumors was still observed in hosts that had been irradiated prior to tumor inoculation. Therefore the inability of the immunogenic tumor to increase the density of LC above the tumor cannot have been due to the irradiation of the hosts prior to tumor injection.

Bergfelt, Bucana, and Kripke [20] have previously reported that the LC density in the epidermis overlying transplantable UV light-induced tumor lines was increased when the tumor was transplanted into syngeneic or athymic mice. They also observed an increased density in the epidermis above tumors that were regressing when transplanted into pre-immunized mice. Whereas Bergfelt et al examined progressor tumor lines that will grow in normal syngeneic mice we have extended these observations to include an immunogenic tumor line. However, the results of both of our experimental approaches found no clear relationship between anti-tumor immunity and LC density above the tumor.

We also examined the effects of UV irradiation on the density of LC overlying the tumors. Even in the presence of continuing UV irradiation, which reduced the LC density, there was an increased density of LC in the epidermis overlying the non-immunogenic but not the immunogenic tumors. Hence, attraction of LC to the tumors was not abrogated by UV irradiation.

To examine whether similar changes in Ia$^+$ cells were occurring within the tumor, frozen tumor sections were examined. One of the tumors, T89, expressed Ia when grown in immunocompetent but not athymic mice, indicating that Ia may have been induced by the host immune system. This is in agreement with previous observations that some but not all human squamous cell carcinomas express class II MHC glycoproteins [21]. The function of these molecules on the tumor cells is unclear, however, T-cell activation requires both antigen presentation in association with class II MHC molecules and co-stimulatory factors [22]. It is possible that these class II MHC molecules on the surface of tumor cells, in the absence of co-stimulatory factors, downregulate T cells, making this tumor non-immunogenic. It has previously been observed that class II MHC-positive keratinocytes induce unresponsiveness, possibly due to an absence of accessory signals [23].

Similar results were obtained for Ia$^+$ cells infiltrating the tumors as for the epidermis overlying the tumor. The non-immunogenic tumors were infiltrated with larger numbers of Ia$^+$ cells than the immunogenic tumor when transplanted into either syngeneic immunocompetent or incompetent mice. Whereas it is unlikely that all of these infiltrating Ia$^+$ cells were LC, the same pattern of changes in Ia$^+$ cells occurred within the tumor as in the epidermis overlying the tumor. Continuous UV irradiation during tumor growth decreased the density of these cells, which has not been previously reported.

In athymic mice, whereas T7 was infiltrated with large numbers of Thy-1$^+$ cells, T79 tumors contained an intermediate number and T89 very few cells, which does not correspond with the pattern of

Table III. Thy-1$^+$ Cells Infiltrating Tumors 4 Weeks Following Inoculation into UV Irradiated and Unirradiated Skh:HR-1 or Unirradiated Athymic Mice

Tumor Line	Mice	UV Radiation	Mean (SD)	N	p[a]	p[b]	p[c]	p[d]
T7	Skh:HR-1	No UV	275 (66)	8				
T7	Skh:HR-1	UV-term	217 (39)	8	ns			
T7	Skh:HR-1	UV-cont	414 (133)	8	<0.01	<0.001		
T79	Skh:HR-1	UV-term	37 (8)	5			<0.001	
T79	Skh:HR-1	UV-cont	95 (34)	4		ns	<0.001	
T89	Athymic	No UV	5 (1)	3			<0.001	
T7	Athymic	No UV	143 (19)	4				
T79	Athymic	No UV	57 (36)	7			<0.001	<0.05

[a] Statistical comparison with unirradiated mice (ANOVA).
[b] Statistical comparison of UV terminating with UV continuing (ANOVA).
[c] Statistical comparison with T7 tumor line (ANOVA).
[d] Statistical comparison with T89 tumor line (ANOVA).

Table IV. Ratio of CD4/CD8$^+$ Cells Infiltrating T89, T7, and T79 Tumors 4 Weeks Following Inoculation into UV Irradiated and Unirradiated Skh:HR-1 Mice

Tumor Line	UV Radiation	Mean (range)	N	p[a]
T89	No UV	1.5 (0.3–2.5)	5	ns
T89	UV-term	0.9 (0.5–1.3)	5	
T89	UV-cont	0.7 (0.4–1.1)	7	ns
T7	No UV	1.7 (0.4–6.0)	8	ns
T7	UV-term	1.0 (0.2–1.6)	8	
T7	UV-cont	3.1 (0.7–6.5)	8	ns
T79	UV-term	4.0 (1.1–10.2)	5	
T79	UV-cont	14.4 (6.2–37.3)	4	ns

[a] Statistical comparison with UV term (Mann-Whitney U test).

Ia$^+$ cells infiltrating these tumors. Athymic mice contain large numbers of epidermal Thy-1$^+$ dendritic cells [24], and we confirmed this in our athymic mice. It is possible that the Thy-1$^+$ cells infiltrating the tumors in our athymic mice are epidermal Thy-1$^+$ dendritic cells.

We also observed that the number of Thy-1$^+$ cells infiltrating the tumors was increased by continual UV irradiation during the period of tumor growth. As far as we are aware, this has not been previously reported but again differs from the pattern of infiltration by Ia$^+$ cells.

The immunogenic T79 tumors contained an increased ratio of CD4/CD8 infiltrating cells compared to the non-immunogenic tumors, indicating that there may have been an inverse relationship between CD4/CD8 ratio and Ia$^+$ cell density. However, UV light had no effect on the CD4/CD8 cell ratio whereas it decreased the LC density. Hence, the pattern of T-cell infiltration of tumors shows no clear relationship with the ability of these tumors to attract LC.

There is no clear relationship between immunogenicity of the tumors, immunocompetence of the hosts, or T-cell infiltration of the tumors and LC infiltration of the tumors. Hence, our data are most consistent with the suggestion that some squamous cell carcinomas, such as T89 and T7 but not T79, produce a cytokine that is chemotactic for LC.

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